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A novel hypobromous acid scavenging activity assay using *p*-cresol as a spectrofluorometric probe

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Abstract: In this study, a novel spectrofluorometric assay based on *p*-cresol (4-methyl phenol) probe is developed for the measurement of HOBr scavenging activity. It is the first study involving the use of a *p*-cresol probe for the determination of the HOBr scavenging activity of biothiols. While the *p*-cresol probe (λ_{ex} = 260 nm, λ_{em} = 305 nm) has fluorescence characteristics, its brominated derivatives emerging at the end of the oxidation reaction with HOBr do not show fluorescence. The initial fluorescence intensity of the *p*-cresol probe is decreased in the presence of the brominating agent, HOBr, and this decrease is lower in the presence of HOBr scavenging antioxidants. The scavenging activities of biothiols tested with respect to the developed method decrease in the following order: penicillamine > N-acetyl cysteine > L-glutathione (reduced) > cysteamine > homocysteine > glutathione ethyl ester > cysteine > 1,4-dithiothreitol > lipoic acid > methionine. Penicillamine (IC_{50} = 10.12 μM) was the most effective HOBr-scavenger among the tested biothiols. The results obtained with the developed method for biothiols and some pharmaceutical samples were statistically compared (using ANOVA) to those found by the reference methods (KI/taurine and UPLC). The advantage of the proposed method over the KI/taurine assay was demonstrated.

Key words: Spectrofluorometric method, hypobromous acid scavenging, *p*-cresol probe, biothiols

1. Introduction

Hypobromous acid (HOBr) is regarded as a reactive bromine species (RBS) with effective antibacterial properties among endogenous reactive oxygen species (ROS).[1] It is thought to be a key component of the neutrophil defense system. Eosinophil peroxidase (EPO) present in eosinophils and macrophages catalyzes the reaction between hydrogen peroxide and bromide ions to form HOBr.

$$Br^- + H_2O_2 + H^+ \xrightarrow{EPO} HOBr + H_2O$$

HOBr can be obtained in the laboratory as a result of the reaction of sodium hypochlorite with bromide ion. In order to inhibit HOBr degradation and HBr formation, a slight stoichiometric excess of the halide (Br^-/HOCl: 1.1:1.0) is used in the reaction.[2]

$$HOCl + Br^- \rightarrow HOBr + Cl^-$$

Although HOBr formation plays a critical role in the immune system, excessive or misplaced generation can cause tissue damage, leading to a wide range of diseases (i.e. cancers, cystic fibrosis, inflammatory bowel

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disease, arthritis, kidney disease, neurodegenerative conditions, asthma).[3] HOBr is a two-electron oxidant, with a standard reduction potential of 1.13 V,[4,5] and reacts rapidly with biological macromolecules (i.e. proteins, lipids, and DNA). Although the production of HOCl and HOBr is an essential part of the innate immune defense, HOBr is more reactive than HOCl.[5] Its reaction with proteins results in bromamines, while reaction with lipids results in bromohydrins.[6]

Due to their participation in the maintenance of cellular redox status and its alterations, biothiols (glutathione (GSH), homocysteine (Hcys), cysteine (Cys), etc.), which play a crucial role in biological systems, are among the target molecules for HOBr. For this reason, determination of HOBr scavenging activity has attracted great interest. Despite this necessity, comprehensive studies on the HOBr scavenging activity measurement of biothiols are lacking. The TNB (2-nitro-5-thiobenzoate)/DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) assay, which is frequently used for the determination of HOBr scavenging activity, is open to interferences due to the fact that biothiols directly reduce DTNB to TNB. TNB-anion is formed as the product of the thiol-exchange reaction of DTNB with free thiols and allows the detection of thiols at micromolar concentrations by its strong absorption at 412-nm wavelength.[7] Therefore, DTNB resulting from the oxidation of TNB with HOBr is converted back into TNB by the biothiol found in the reaction medium, resulting in an overestimation of the scavenger (biothiol) in the HOBr scavenging activity assay. On the other hand, the thiocyanate anion (SCN^-), which is present in significant concentrations in human biological fluids, is one of the most effective scavengers of HOBr, and at the end of the rapid oxidation reaction it turns into a hypothiocyanate ($OSCN^-$) anion, which itself is a moderating oxidant.[8] Therefore, possible SCN^-/$OSCN^-$ interference in spectroscopic tests should also be considered when determining the HOBr scavenging activity of physiological fluids. If the analyst looks at possible bromination products, 3-bromotyrosine and 3,5-dibromotyrosine, which are formed as a result of reaction with HOBr, are considered to be the key indicators of oxidative damage resulting from reactive bromine species occurring in proteins. These products are usually detected by HPLC and GC/MS methods.[9] These methods are time-consuming and expensive, requiring costly equipment that is not available in routine laboratories. A few fluorogenic probes were developed to monitor HOBr formation.[10,11] Hence, it is reasonable to suppose that there is a requirement for novel HOBr scavenging activity assays for biothiols, overcoming the above restrictions.

In the present work, a new spectrofluorometric method based on a *p*-cresol (4-methyl phenol; λ_{ex} = 260 nm, λ_{em} = 305 nm) probe was developed for the measurement of HOBr scavenging activity of biothiols (GSH, Cys, Hcys, etc.) and complex pharmaceutical samples (mentopin, primene, brunac, etc.). The products formed from the reaction of the *p*-cresol probe with HOBr along with the change in probe concentration in the presence and absence of HOBr scavengers were also determined by ultra performance liquid chromatography (UPLC) to validate the developed fluorometric method. In addition, the findings for biothiols and pharmaceutical samples were compared to those of the KI/taurine reference method. In conclusion, the developed fluorometric method is thought to be a good alternative for the rarely existing HOBr scavenging activity assays in the literature.

2. Results and discussion

A novel method for the evaluation of HOBr scavenging activity of biothiols is described, based on the fluorometric determination of the remaining*p*-cresol after reaction with HOBr in the presence and absence of scavengers.

HOBr, generated by mixing HOCl with KBr in equal volumes, attacks both the *p*-cresol probe and potential scavengers. At the end of this competitive reaction between probe and scavenger, *p*-cresol gives rise to basically nonfluorescent oxidation products (i.e. mono-, di-, tri-bromo cresols), resulting in a decrease in

the fluorescence of *p*-cresol.[3] Probably the most advantageous point in the selection of the *p*-cresol probe is its selectivity toward HOBr, as the standard reduction potential of HOBr is 1.13 V[5] while the oxidation of *p*-cresol was found to occur at 0.972 V on an unmodified glassy carbon electrode (GCE) at a concentration of 2.5×10^{-4} M.[12] *p*-Cresol, at a sufficiently high oxidation potential, is expected to show negligible activity as a potential scavenger of peroxyl radical, singlet oxygen, and hydrogen peroxide, having quite lower potentials as alternative oxidizing agents.[13]

It has been reported in the literature that *p*-cresol is a highly fluorescent substance (λ_{ex} = 260 nm, λ_{em} = 305 nm).[14] Under the selected conditions, the molar fluorescence coefficient of *p*-cresol was 4.96×10^6 M^{-1} cm^{-1} and the linear concentration range was 1.64×10^{-6}–5.0×10^{-5} M. The limit of detection (LOD) was calculated using the equation LOD = $3s_{bl}/m$, where s_{bl} is the standard deviation of a blank signal (fluorescence intensity) and m is the slope of the calibration line. The LOD for the *p*-cresol probe in the fluorometric assay was 3.70×10^{-7} M. The second-order rate constant for the reaction of HOBr with *p*-cresol was reported as 2.1×10^8 M^{-1} s^{-1}.[15]

2.1. Testing of reaction mixtures with the fluorometric *p*-cresol assay

The *p*-cresol probe had an initial fluorescence intensity at 304 nm (Figure 1a), which was not influenced in the presence of the HOBr scavengers (Figure 1b). GSH competitively scavenges a part of the HOBr in the reaction system, *p*-cresol undergoes less bromination in the presence of scavenger, and consequently, the fluorescence intensity due to intact *p*-cresol increases (Figure 1c) compared to that of the reference. The tested HOBr scavengers such as GSH do not have fluorescence intensity at 304 nm (Figure 1d). It is apparent that neither bromination products of *p*-cresol (i.e. *p*-cresol + HOBr (Figure 1e)) nor bromination products of GSH upon HOBr attack (GSH + HOBr; Figure 1f) exhibit a fluorescence intensity at 304 nm, meaning that competition of GSH with the *p*-cresol probe for HOBr can be followed simply by observing the changes in the fluorescence intensity of the probe without interference. In other words, the only constituent giving rise to fluorescence intensity in the system is the *p*-cresol probe (Figures 1a–1e).

Fluorescence intensity of the mixture was measured as a function of time. There was no significant change

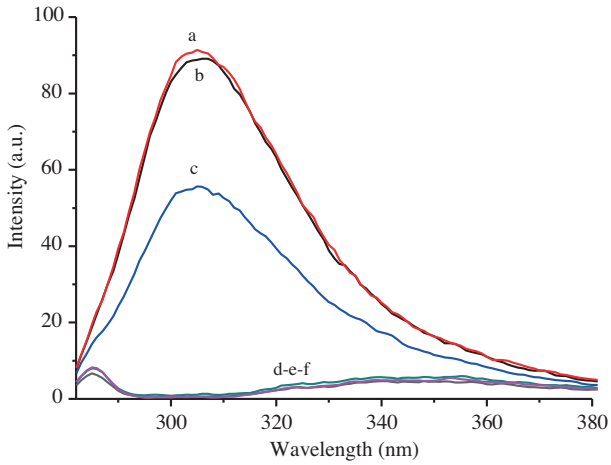

Figure 1. Fluorescence spectra of the reaction mixture (λ_{ex} = 260 nm) (a) *p*-cresol; (b) *p*-cresol + GSH; (c) *p*-cresol + GSH + HOBr; (d) GSH; (e) *p*-cresol + HOBr; (f) GSH + HOBr.

in intensity of solutions within the 0–30-min time interval (Figure 2). Therefore, fluorescence intensity of the reaction mixture could be measured without delay. In the first minutes of the reaction, an intensity difference between the presence and absence of a scavenger was sufficient to calculate a scavenging ratio.

Figure 3 shows the fluorescence spectra of p-cresol recorded in a reaction mixture with varying N-acetyl-L-cysteine (NAC) concentrations. The maximal decrease in fluorescence intensity of p-cresol occurred in the reference (blank) because of the reaction of p-cresol with HOBr, resulting in its oxidation to nonfluorescent products. When the probe was subjected to bromination in the presence of a scavenger, the relative increase in fluorescence from baseline level was proportional to the scavenging ability of the competitive scavenger. The fluorescence intensities of p-cresol in the presence of NAC at concentrations of 2.22–11.1 μM increased with increasing concentration of scavenger (Figure 3). The scavenging ratio of biothiols can be calculated by using the fluorescence intensity of p-cresol recorded in the presence and absence of scavengers at varying concentrations.

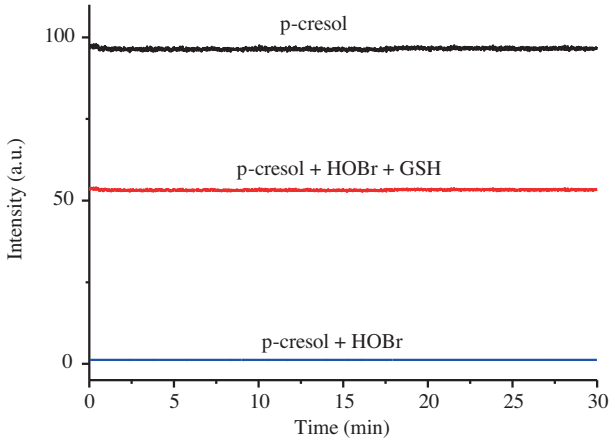

Figure 2. Fluorescence intensity vs. incubation time curves of p-cresol alone and p-cresol subjected to HOBr in the absence (reference) and presence of a scavenger (GSH).

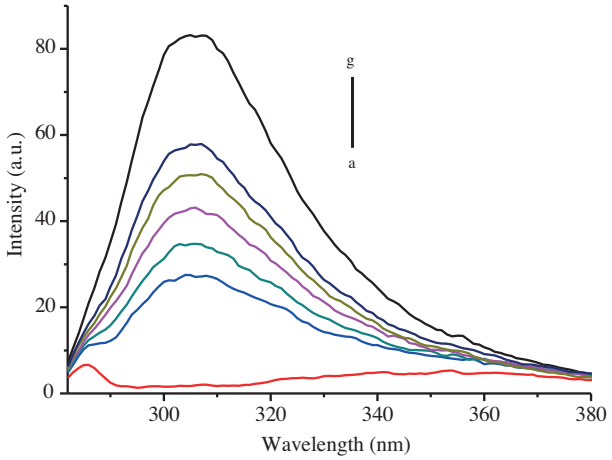

Figure 3. Fluorescence spectra of the remaining p-cresol in the absence and presence of NAC (a: 0 μM NAC (reference), b: 2.22 μM NAC, c: 4.44 μM NAC, d: 6.66 μM NAC, e: 8.88 μM NAC, f: 11.1 μM NAC, g: 16.4 μM p-cresol).

2.2. Determination of the HOBr scavenging activity of biothiols

Taurine, a sulfur-containing amino acid, is a major scavenger for hypohalous acids (HOX; x = Cl or Br). The product of the reaction between taurine and HOBr is taurine bromamine (TauBr),[16] which has a molar extinction coefficient of 430 M^{-1} cm^{-1} at 288 nm.[17,18] The quantification of taurine bromamine at 288 nm is not sufficiently sensitive because of the low extinction coefficient. Its concentration can be sensitively determined by using TNB-anion or iodide (I^-).[19] Taurine bromamine oxidizes 2 moles of TNB to 1 mole of DTNB, or 2 moles of I^- to 1 mole of I_2. TNB can be determined by measuring its absorbance at 412 nm, using an extinction coefficient of 13,600 M^{-1} cm^{-1}.[20] The I_2 concentration was determined in the presence of excess I^- as I_3^- at 350 nm (ε = 22,900 M^{-1} cm^{-1}).[21] On the other hand, use of TNB is not suitable to determine the HOBr scavenging activity of biothiols. DTNB, formed at the end of the reaction between HOBr and TNB, can be reduced back to TNB by means of the remaining biothiol in the reaction medium, possibly giving rise to overestimated results. Moreover, the limited stability of TNB is one of the drawbacks of this method.[22]

Thiols react with hypohalous acids (HOX; x = Cl or Br) through the generation of an unstable sulfenyl halide (R–S–X), which readily rearranges to a sulfenic acid.[23]

$$R - S^- + HOBr \rightarrow R - S - Br + OH^-$$

$$R - S - X + H_2O \rightarrow R - S - OH + HX$$

Cysteine is an aminothiol containing three reactive centers such as carboxylic acid, amino group, and the sulfur center. Darkwa et al. reported that only the sulfur center of cysteine is responsible for scavenging activity despite the carboxylic acid and amino groups. In contrast to taurine, the amino group of cysteine is inert and no reactivity has been detected at the nitrogen center.[24]

HOBr scavenging activities (IC_{50} (μM)) of biothiols were assayed by *p*-cresol and KI/taurine assays (Table).[25] In both methods, penicillamine showed the highest HOBr scavenging activity. Biothiols that do not bear a free sulfhydryl group, such as lipoic acid and methionine, showed lower HOBr scavenging activity than other biothiols containing a sulfhydryl group. The descending order of HOBr scavenging activity in the *p*-cresol assay was as follows: penicillamine > N-acetyl-L-cysteine > L-glutathione reduced > cysteamine > homocysteine > glutathione reduced ethyl ester > cysteine > 1,4- dithioerythritol > lipoic acid > methionine.

Table. HOBr scavenging activity of various biothiols measured by the *p*-cresol assay in comparison to the KI/taurine assay (IC_{50} values were calculated with respect to Eqs. (1) and (2); $N = 4$ or 5 data points).

Biothiol	IC_{50} value with respect to *p*-cresol assay (μM)	IC_{50} value with respect to KI/taurine assay (μM)
Penicillamine	10.12 ± 0.5	9.79 ± 0.09
N-acetyl-L-cysteine	12.31± 0.4	11.23± 0.2
L-glutathione (reduced)	12.88 ± 0.3	12.03 ± 0.2
Cysteamine	13.48 ± 0.8	30.28 ± 1.3
Homocysteine	13.98 ± 0.7	9.00 ± 0.08
Glutathione reduced ethyl ester	20.33 ± 0.9	16.00 ± 0.6
L-cysteine	20.36 ± 0.6	22.00 ± 0.8
1,4-Dithioerythritol	23.42 ± 0.5	22.91 ± 1.4
Lipoic acid	31.38 ± 0.2	23.38 ± 0.2
L-methionine	34.88 ± 0.6	25.00 ± 0.4

P= 0.05, $F_{exp} = 0.24931$, $F_{crit(table)} = 5.117$, $F_{exp} < F_{crit(table)}$. Data presented in the form of {mean±SD}, N=3.

Cysteamine is known as an excellent scavenger of hydroxyl radical and hypochlorous acid.[26] Özyürek et al. reported the HOCl scavenging activity of cysteamine in terms of the IC_{50} values found with resorcinol and KI/taurine assays as 13.51 μM and 33.30 μM, respectively.[13] Similarly, the HOBr scavenging activity of cysteamine (as IC_{50} values) found with *p*-cresol and KI/taurine assays was 13.48 μM and 30.28 μM, respectively. The IC_{50} values of cysteamine obtained with the KI/taurine assay for both reactive species (33.30 μM for HOCl and 30.28 μM for HOBr) were close to each other. However, these values were higher than the IC_{50} values found with the resorcinol (13.51 μM) and *p*-cresol (13.48 μM) assays. Morakinyo et al. reported that cysteamine can be oxidized with aqueous bromine to taurine and its N-brominated products, and that taurine formation from cysteamine can be readily reversed by iodide (i.e. to form molecular iodine).[27] In other words, in the KI/taurine assay of cysteamine, more iodine is produced than expected, making cysteamine a seemingly less effective scavenger of HOBr (thereby showing an overestimated IC_{50} value). The *p*-cresol

spectrofluorometric assay of this work correctly assigns a lower IC_{50} value to cysteamine, just as the resorcinol assay previously published.[13] This is perhaps one of the most striking advantages of the proposed assay in determining the true HOBr scavenging activities of biothiols.

Spearman's rank correlation test was employed for comparative IC_{50} values found with the *p*-cresol and KI/taurine assays. The correlation coefficient (R) was calculated as 0.6364. When the same test was repeated by excluding cysteamine, the correlation coefficient went up to 0.90, which indicates a positive relationship between the ranks.

2.3. Comparison of the findings of the *p*-cresol and UPLC assays

Conversion of the *p*-cresol probe to oxidation products and inhibition of this reaction with biothiols were examined by the *p*-cresol and UPLC assays. The chromatograms of the original and remaining *p*-cresol after the reaction with HOBr in the absence and presence of a scavenger (specifically NAC) are shown in Figure 4, where *p*-cresol was identified at a retention time of 4.94 min. The peak area of the *p*-cresol probe was higher in the presence of NAC due to less conversion to oxidation product(s). The peak observed at 5.01 min is thought to be an oxidation product of *p*-cresol showing a decreasing trend with increasing concentration of the scavenger. Results found with both assays correctly exhibited the relative decrease in probe conversion into oxidation product(s) in the presence of NAC (Figures 3 and 4). The amounts of *p*-cresol in the reaction mixture were calculated using the calibration equation ($y = 487c - 650$ ($r = 0.9989$)) drawn as peak area (y) versus concentration (c, in μM). In the absence of scavengers (reference), almost all *p*-cresol probe was converted to oxidation product(s) (Figure 4a). The peak area of the probe increased with increasing concentrations of the scavenger due to less conversion to oxidation products. An increase in the peak area of the probe indicated an increase in HOBr scavenging activity. The HOBr scavenging activities of NAC (as IC_{50} values) found with the *p*-cresol and UPLC assays were 12.31 μM and 12.28 μM, respectively.

2.4. Application of the *p*-cresol assay to pharmaceutical samples

The *p*-cresol assay was successfully applied to pharmaceutical samples containing biothiols and their percentages of scavenging are comparatively depicted in a bar diagram (Figure 5). N-acetyl cysteine is an active ingredient of Mentopin effervescent tablets and Brunac eye drops. The HOBr scavenging activities of Mentopin and Brunac for the *p*-cresol assay were in good agreement with those measured by the KI/taurine assay. However, the HOBr scavenging activity of Primene amino acid solution found with the *p*-cresol assay (64.55%) was distinctly higher than that of the KI/taurine assay (32.79%). The relative standard deviation (%, RSD) for the Primene amino acid solution was calculated as 1.59 in the working concentration range. Primene amino acid solution contains twenty kinds of amino acids like taurine (4.79 mM), cysteine (15.7 mM), and methionine (16 mM). The KI/taurine assay depends on taurine bromination when taurine is in the reaction medium as both probe and scavenger, and hence the negative interference on the inhibition results is inevitable due to excessive formation of taurine bromamine. Scavenging percentage was once again determined by using UPLC method in order to verify the HOBr scavenging activity of Primene amino acid solution. The UPLC result for the scavenging percentage of Primene amino acid solution (62.24%) was found in accordance with that of the *p*-cresol assay, confirming the interference-free applicability of the *p*-cresol spectrofluorometric method to real samples. As Primene amino acid solution contains efficient HOBr scavengers such as valine, lysine, alanine, and glutamic acid in addition to cysteine and methionine, it showed the highest percentage scavenging among the studied pharmaceutical samples.

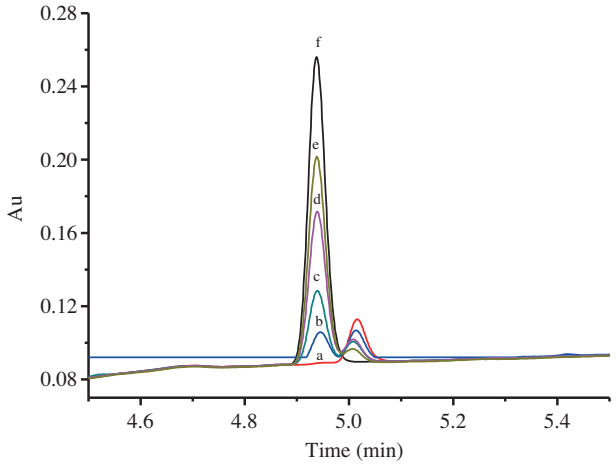

Figure 4. UPLC chromatograms for remaining p-cresol in the absence and presence of NAC (a: 0 μM NAC (reference), b: 4.44 μM NAC, c: 8.88 μM NAC, d: 13.33 μM NAC, e: 17.78 μM NAC, f: 16.4 μM p-cresol).

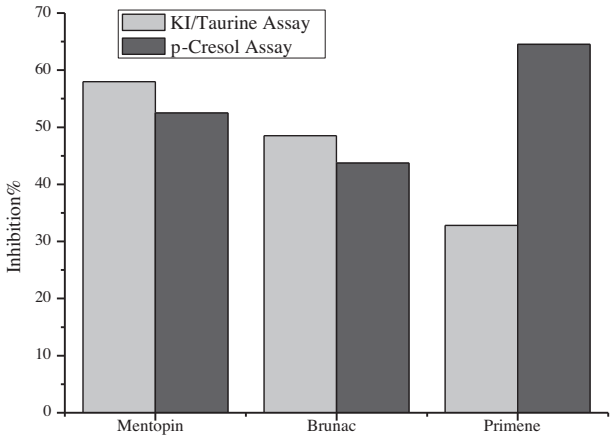

Figure 5. Percentage scavenging of pharmaceutical samples calculated with the p-cresol assay, in comparison to that with the KI/taurine assay.

3. Experimental

3.1. Reagent and apparatus

Chemical substances of analytical reagent grade were obtained from the sources given below: cysteamine, homocysteine, lipoic acid, taurine, potassium bromide, 1,4-dithioerythritol, glutathione (reduced) ethyl ester, ethanol (EtOH): Sigma (Taufkirchen, Germany); penicillamine: Aldrich (Taufkirchen, Germany); formic acid, p-cresol, acetonitrile, sulfuric acid: Sigma-Aldrich (Taufkirchen, Germany); L-glutathione (reduced: GSH), sodium hypochlorite (NaOCl): Merck (Darmstadt, Germany); potassium iodide: Riedel-de Haen (Taufkirchen, Germany); L-cysteine, L-methionine, N-acetyl-L-cysteine (NAC): Fluka (Buchs, Switzerland).

The fluorescence intensity and absorption measurements were performed using a VARIAN (Mulgrave, Victoria, Australia) Cary Eclipse spectrofluorometer and VARIAN Cary 100 UV-Vis spectrophotometer, respectively, in quartz cuvettes.

UPLC analysis was performed using a Waters Acquity (Milford, MA, USA) equipped with a binary solvent manager, sample manager, column thermostat, and diode array detector. An Acquity BEH C18 analytical column (100 $\times$ 2.1 mm, 1.7 μm) (Milford, MA, USA) was used for gradient elution of the p-cresol probe. Data acquisition was accomplished using MassLynx v 4.1 software (Waters, Milford, MA, USA).

3.2. Preparation of solutions

The p-cresol solution (74 μM), KI (2.0 M), and taurine (2-amino ethanesulfonic acid) (150 mM) were all prepared in pure distilled water (Millipore Simpak1 Synergy 185, USA). The HOCl stock solution was prepared by diluting a 0.2% (v/v) solution of NaOCl from commercial solution immediately before use and adjusting the solution pH to 9 with dilute H_2SO_4. Working solution of HOCl was prepared by 3-fold dilution of the stock solution with distilled water. The concentration of HOCl was further determined spectrophotometrically at 292 nm using the molar absorption coefficient of $\varepsilon = 350\ M^{-1}\ cm^{-1}$.[28]

Solution of HOBr was prepared by adding HOCl to a slight molar excess of KBr (4 mM) in water to

prevent HBr formation and consequent HOBr decay.[29,30] Conversion of HOCl to HOBr was monitored by the loss of UV absorption of OCl^- (λ_{max} = 292 nm) and formation of OBr^- (λ_{max} = 329 nm) at pH 9. HOBr and OBr^- were determined spectrophotometrically at 260 nm and 329 nm, respectively, using the molar absorption coefficients ($\varepsilon_{260\,nm}$ = 100 M^{-1} cm^{-1}, $\varepsilon_{329\,nm}$ = 332 M^{-1} cm^{-1}).[2]

All biothiols were freshly prepared in distilled water within the concentration range of 0.1–1.0 mM. The mobile phase constituents for UPLC analysis with gradient elution were 0.01% of formic acid in bidistilled water (A) and 0.01% of formic acid in acetonitrile (B).

The pharmaceuticals (Mentopin, Brunac, and Primene) were purchased from pharmacies in İstanbul. The effervescent tablets (Mentopin) were dissolved and diluted with distilled water. The eye drops (Brunac) and 10% amino acid solution (Primene) were diluted with distilled water. The final pharmaceutical solutions were analyzed for HOBr scavenging activity with both *p*-cresol and KI/taurine methods.

3.3. *p*-Cresol assay

Reaction mixtures contained, in a final volume of 4.5 mL, 1.0 mL of pH 7.4 phosphate buffer (0.2 M), 1.0 mL of *p*-cresol (74 μM), x mL of scavenger at a suitable concentration, and (2.0 – x) mL of water, and the reaction was started by adding 0.5 mL of HOBr (3.91×10^{-4} M). The fluorescence intensity (λ_{ex} = 260 nm, λ_{em} = 305 nm) of the reaction mixture was recorded.

The HOBr scavenging activity was calculated using the following equation, where I_0 is the initial fluorescence intensity of the *p*-cresol probe solution (mean value, I_0 = 81), I_1 (mean fluorescence intensity of the blank = 2), and I_2 of the *p*-cresol probe subjected to HOBr oxidation in the absence and presence of biothiols, respectively:

$$\text{Scavenging ratio } (\%) = 100[(I_2 - I_1)/(I_0 - I_1)] \quad (1)$$

The IC_{50} (antioxidant concentration capable of inhibiting 50% of the bromination reaction of *p*-cresol or of scavenging 50% of HOBr) values of biothiols were determined using a *p*-cresol probe, by means of a plot of the scavenging percentage as a function of the molar concentration of biothiols.

3.4. KI/taurine assay

The spectrophotometric KI/taurine reference method[25] is based on the oxidation of I^- to I_2 by taurine bromamine. The I_2 concentration was determined spectrophotometrically at 350 nm ($\varepsilon = 2.29 \times 10^4$ $M^{-1}s^{-1}$).[31] To a test tube were added 1.0 mL of phosphate saline buffer (pH = 7.4), (2.0 – x) mL of distilled water, 0.5 mL of taurine, x = 0 – 0.9 mL of scavenger solution at a suitable concentration, and 0.3 mL of HOBr in this order. The solution was mixed and 0.5 mL of potassium iodide was added. A yellow coloration developed and the absorbance was read at 350 nm.

The scavenging ratio of biothiols (%) was calculated using the following equation:

$$\text{Scavenging ratio } (\%) = 100[(A_o - A)/A_o], \quad (2)$$

where A_o (mean absorbance of the blank = 1.1) and A are the absorbances of the system in the absence and presence of biothiols, respectively.

3.5. UPLC assay

To analyze the *p*-cresol probe, the mobile phase consisted of two solvents, i.e. 0.01% of formic acid in bidistilled water (A) and 0.01% of formic acid in acetonitrile (B).[17] The analysis were performed by using gradient elution: (Flow rate = 0.40 mL/min; column temperature: 25 °C): 0 min 90% A – 10%, B; 2 min 85% A – 15% B (slope 1.0); 3 min 80% A – 20% B (slope 1.0); 4 min 70% A – 30% B (slope 1.0); 6 min 65% A – 35% B (slope 1.0); 8 min 55% A – 45% B (slope 1.0); 10 min 40% A – 60% B (slope 1.0); 12 min 90% A – 10% B (slope 1.0); 15 min 90% A – 10% B (slope 1.0). Detection wavelength was set from 190 to 400 nm for the photodiode array detector. All injected solutions were stored at 4 °C in the autosampler.

The scavenging ratio of biothiols (%) was calculated using the following equation:

$$\text{Scavenging ratio (\%)} = 100[(A_2 - A_1)/(A_o - A_1)], \quad (3)$$

where A_1, A_2, and A_0 are the peak areas of the *p*-cresol probe in the absence and presence of scavenger and at initial concentration (i.e. prior to bromination) in the reaction mixture, respectively.

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